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# RNA-seq reveals the critical role of CspA in regulating *Brucella melitensis* metabolism and virulence

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*Brucella melitensis* is a facultative intracellular bacterium that replicates within macrophages. The ability of *Brucella* to survive and multiply in the hostile environment of host macrophages is essential for its virulence. The cold shock protein CspA plays an important role in the virulence of *B. melitensis*. To analyze the genes regulated by CspA, the whole transcriptomes of *B. melitensis* NIΔ*cspA* and its parental wild-type strain, *B. melitensis* NI, were sequenced and analyzed using the Solexa/Illumina sequencing platform. A total of 446 differentially expressed genes were identified, including 324 up-regulated and 122 down-regulated genes. Numerous genes identified are involved in amino acid, fatty acid, nitrogen, and energy metabolism. Interestingly, all genes involved in the type IV secretion system and LuxR-type regulatory protein VjbR were significantly down-regulated in NIΔ*cspA*. In addition, an effector translocation assay confirmed that the function of T4SS in NIΔ*cspA* is influenced by deletion of the *cspA* gene. These results revealed the differential phenomena associated with virulence and metabolism in NIΔ*cspA* and NI, providing important information for understanding detailed CspA-regulated interaction networks and *Brucella* pathogenesis.

***Brucella melitensis*, cold shock protein, transcriptome, virulence**

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## INTRODUCTION

*Brucella melitensis* is a facultative intracellular pathogen that resides and replicates within specialized compartments of macrophages (Martirosyan et al., 2011). The ability of brucellae to survive within macrophages is essential for their virulence (Celli and Gorvel, 2004). In the past few years, hundreds of virulence-related genes have been identified by transposon mutagenesis (Wu et al., 2006), *in vivo*-induced antigen technology (Lowry et al., 2011), DNA microarray hybridization (Tian et al., 2013), and bioinformatics (He, 2012), which include lipopolysaccharide (Seleem et al., 2008), the type IV secretion system T4SS (Seleem et al., 2008; Martirosyan et al., 2011), the quorum-sensing-related transcriptional regulator VjbR (Delrue et al., 2005), the BvrR/BvrS two-component regulatory system (Sola-Landa et al., 1998; Martinez-Nunez et al., 2010), and the periplasmic cyclic β-1,2 glucans (Briones et al., 2001), among others. Interestingly, most of these genes are not direct virulence factors, but are transcription regulators, such as VjbR which can control the expression of virulence factors to affect *Brucella* virulence (Delrue et al., 2005). Novel transcription regulators and regulatory RNAs related to the virulence of *Brucella* were recently identified, including MucR (Dong et al., 2013; Mirabella et al., 2013), GntR (Haine et al., 2005), NnrA (Haine et al., 2006),

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AbcR1, and AbcR2 (Caswell et al., 2012).

Cold-shock proteins exist in most organisms; among these proteins, the CspA family has been studied in detail in *Escherichia coli* and *Bacillus subtilis* (Ermolenko and Makhatadze, 2002; Phadtare, 2004). In *E. coli*, CspA protein has been shown to act as an RNA chaperone (Jiang et al., 1997) that significantly regulates its own gene expression (Bae et al., 1997) as well as flagella gene expression (Phadtare and Inouye, 2004), which is an important virulence factor in *Brucella* (Fretin et al., 2005). Our previous experiments demonstrated that NIΔ*cspA*, a *cspA* mutant of the virulent strain *B. melitensis* NI, was attenuate in mice and showed reduced survival in phagocytic cells. Moreover, unlike its parental strain, *B. melitensis* NI, the NIΔ*cspA* mutant showed increased sensitivity to acidic and $H_2O_2$ stresses, particularly during the mid-log phase (Wang et al., 2014). To further understand the influence of CspA on gene expression in the *B. melitensis cspA* mutant, the whole transcriptome of NIΔ*cspA* was sequenced using the Solexa/illumina platform and then the *cspA*-targeted genes of interest were confirmed by reverse transcriptase-polymerase chain reaction and quantitative real-time PCR (qRT-PCR) analysis. Through highly efficient sequencing of complementary DNAs (cDNAs) that can be reverse-transcribed from RNAs, RNA-seq technology has many advantages compared to microarray technology in whole-genome gene expression analysis (Pinto et al., 2011). RNA-seq does not require probe sequences and has a greater dynamic range for measuring very low or very high gene expression levels (Croucher and Thomson, 2010). The power of RNA-seq has been demonstrated in the transcriptomics studies of *Brucella* (Wang et al., 2011; Rodriguez et al., 2012) and many other bacteria (Croucher and Thomson, 2010; Pinto et al., 2011). Therefore, we used RNA-seq to provide fundamental gene-level evidence and detailed gene expression profiles regulated by CspA in *Brucella.*

## RESULTS

### *cspA* differentially regulated 446 genes in *Brucella*

Our previous studies showed that CspA contributes to the virulence of *B. melitensis*. However, the global effects of CspA on the gene transcription pattern of *B. melitensis* remain unclear. Thus, cDNA samples from NIΔ*cspA* and *B. melitensis* NI were sequenced. A total of 6,511,928 reads for NIΔ*cspA* and 6,439,528 reads for *B. melitensis* NI were determined. After quality evaluation using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the clean reads were matched to the genome of *B. melitensis* NI using the alignment program SOAP. Statistical analysis revealed a total of 446 genes that were differentially expressed in NIΔ*cspA* versus in *B. melitensis* NI. Among these genes, 122 genes were down-regulated and 324 genes were up-regulated in NIΔ*cspA* compared to in the NI control (the complete list of differentially expressed genes in NIΔ*cspA* versus *B. melitensis* NI is shown in Table S1).

### Functional classification of differentially expressed genes by COG

COG is a database that builds on coding proteins with complete genomes. The purpose of COG is to serve as a platform for functional classification and annotation for many new sequences (Lin et al., 2011). Because there is no COG database of *B. melitensis* NI, the COG database of *B. melitensis* 16M was used to analyze the 446 differentially expressed genes in NIΔ*cspA*, which found 372 out of 446 genes in the *B. melitensis* 16M genome. Next, the 372 genes were aligned to COG and assigned to 21 COG classifications. Among the 21 COG categories, the cluster for amino acid transport and metabolism ($n$=56, 15%) was the largest group, followed by energy production and conversion ($n$=24, 6.45%), translation, ribosomal structure, and biogenesis ($n$=23, 6.18%), and cell wall/membrane/envelope ($n$=23, 6.18%), but the percentages of chromatin structure and dynamics, cell cycle control, cell division, chromosome partitioning, and replication, recombination, and repair, were <1.00% (Figure 1). None of the genes were assigned to RNA processing and modification, extracellular structures, nuclear structure, and cytoskeleton. Interestingly, genes assigned to the intracellular trafficking, secretion, and vesicular transport and translation, ribosomal structure, and biogenesis groups were among the down-expressed genes in NIΔ*cspA*.

### Regulation of various pathways

KEGG pathway analysis was performed using DAVID Bioinformatics Resource (Jiao et al., 2012). In the up-regulated genes group, 93 genes were enriched in metabolism pathways including various amino acid and lipid metabolism pathways. Additionally, many up-regulated genes included ABC transporters, two-component systems, and flagella assembly pathways. Among the down-regulated genes, the bacterial secretion system pathway was the top pathway containing 11 genes, followed by oxidative phosphorylation, ABC transporter pathways, and flagella assembly pathways. KEGG pathway analysis demonstrated that CspA is an important regulator that affects the expression of most *B. melitensis* metabolic- and virulence-related genes.

### Regulation of metabolism in *B. melitensis*

Transcriptome analysis indicated that a large number of genes associated with metabolism were differently expressed in NIΔ*cspA*. These included BMNI_II0026-0028, BMNI_II0309, BMNI_II0310, BMNI_II0357, BMNI_II0358, BMNI_II0604, BMNI_II0794, BMNI_II0894-0896, and BMNI_II1119 which participate in amino acid transport and metabolism, BMNI_II0741 and BMNI_II0742 encoding acyl-COA dehydrogenase participate in fatty acid

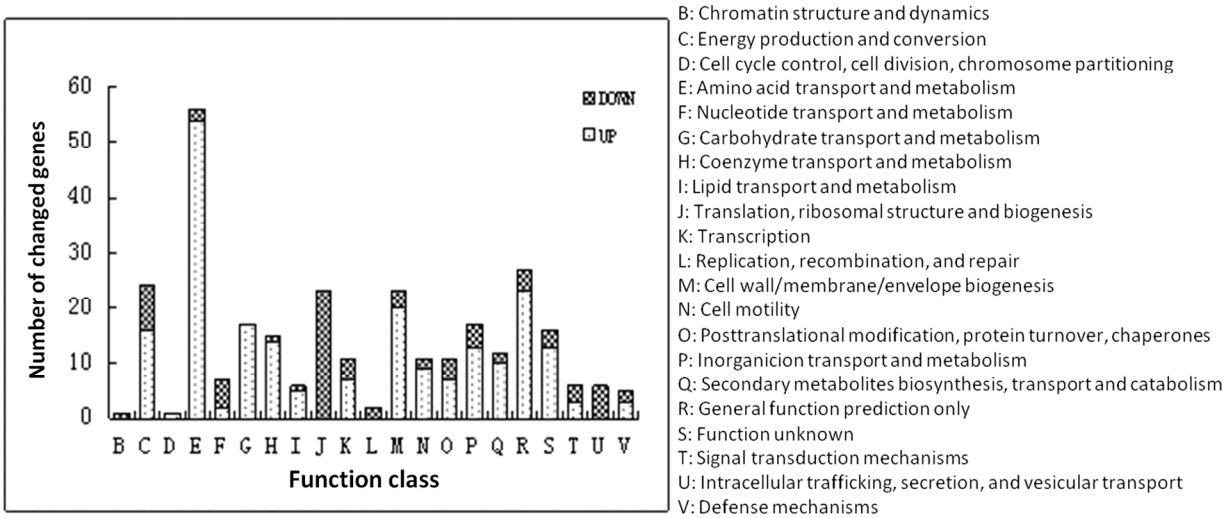


**Figure 1** Distribution of significantly regulated genes classified according to the clusters of orthologous groups (COGs). Only genes that were up- or down-regulated by ⩾2.0-fold are shown.

metabolism. In addition, two genes, BMNI_II0741 and BMNI_I1231, are involved in pyruvate metabolism, which plays an important role in balancing energy metabolism (Martin et al., 2005), and were both up-regulated in NIΔ*cspA*. Interestingly, eight genes, BMNI_II0247, BMNI_II0261, BMNI_II0262, and BMNI_II0284-0288 encoding nitrite reductase that participate in the denitrification pathway, were all up-regulated in NIΔ*cspA*. Moreover, some of these denitrification genes have been related to virulence of *Brucella* in mice (Loisel-Meyer et al., 2006).

### Regulation of *Brucella* virulence-related factors

One of the remarkable findings of the RNA-seq analysis was that the genes associated with virulence were significantly differentially expressed. Nearly all *virB* operon genes (BMNI_II0056, BMNI_II0057, BMNI_II0058, BMNI_II0059, BMNI_II0061, BMNI_II0062, and BMNI_II0064) encoding T4SS were significantly down-regulated in NIΔ*cspA*. Six genes related to flagellar assembly, including *flg*A (BMNI_II0148), *fli*C (BMNI_II0145), *fli*E (BMNI_II0146), *flg*G (BMNI_II0147), *flg*H (BMNI_II0151), *flg*I (BMNI_II0149), were up-regulated, while one gene *flh*B (BMNI_II0117) was down-regulated in NIΔ*cspA*. Notably, one transcriptional regulator gene BMNI_II0115 (LuxR-type regulatory protein, VjbR) was significantly down-regulated in NIΔ*cspA*, VjbR was previously found to directly activate the secretion system *virB* operon and flagellar genes (Delrue et al., 2005). It has also been reported that some OMPs of *B. melitensis* are involved in virulence. In our study, expression of the outer membrane protein Omp31 coding gene BMNI_II0387 was significantly decreased. In addition, six additional transcriptional regulators, including the GntR family transcriptional regulator (BMNI_II0373, BMNI_II0353, and BMNI_I1651), Crp/ FNR family transcriptional regulator (BMNI_II0377), TETR family transcriptional regulator (BMNI_I0306), and Rrf2 family transcriptional regulator (BMNI_II1061), were all up-regulated in NIΔ*cspA*.

### Growth characteristics of *cspA* deletion mutant in minimal medium

Our transcriptome analysis results indicated that the CspA control elements are involved in the metabolism of *B. melitensis*. To determine whether CspA affects metabolism, NIΔ*cspA*, NIΔ*cspA* PBBR*cspA*, and *B. melitensis* NI were grown in minimal media. The mutant NIΔ*cspA* showed reduced growth in minimal media compared with the parent strain NI, whereas introduction of pBBR*cspA* restored the growth of NIΔ*cspA* to the level of the parental strain (Figure 2).

### RT-PCR verification of RNA-seq results of selected *Brucella* genes

To validate the data generated from the RNA-seq experiment, we used RT-qPCR assays to detect the transcript levels of 14 genes down-regulated in NIΔ*cspA* and of 4 genes up-regulated in NIΔ*cspA*. Data were normalized by the $2^{-\Delta\Delta C_t}$ method using the 16S rRNA gene of *B. melitensis* as a reference. Of the 446 differentially expressed genes detected in our RNA-Seq analysis, 18 genes were selected based on gene functions and virulence factor roles. Transcriptional data for these selected genes showed good consistency for both RNA-seq and qRT-PCR (Table 1), and 14 genes were down-regulated and 4 genes were up-regulated in NIΔ*cspA*. These results confirmed the good correlation between RNA-seq and qRT-PCR data, thus validating the model.

**Table 1** Transcriptional level of *B. melitensis* genes obtained by quantitative real-time PCR (qRT-PCR).

| *B. melitensis* NI (ORF) | *B. melitensis* 16M (ORF) | Gene predicted function | Length of PCR products (bp) | RT-PCR | RNA-seq |
|---|---|---|---|---|---|
| BMNI_II0062 | BMEII0029 | P-type DNA transfer protein VirB5 | 138 | –2.1 | –5.5 |
| BMNI_II0064 | BMEII0027 | type IV secretion system protein VirB3 | 113 | –2.0 | –5.5 |
| BMNI_II0063 | BMEII0028 | type IV secretion system protein VirB4 | 181 | –2.0 | –5.5 |
| BMNI_II0115 | BMEII1116 | Regulatory protein LuxR | 188 | –1.9 | –5.4 |
| BMNI_II0061 | BMEII0030 | Protein VirB6 | 114 | –1.7 | –4.8 |
| BMNI_II0056 | BMEII0035 | P-type DNA transfer ATPase VirB11 | 85 | –1.6 | –4.1 |
| BMNI_II0058 | BMEII0033 | P-type conjugative transfer protein VirB9 | 256 | –1.5 | –3.7 |
| BMNI_II0057 | BMEII0034 | Type IV secretion system protein virB10 | 251 | –1.6 | –3.7 |
| BMNI_II0059 | BMEII0032 | Type IV secretion system protein virB8 | 118 | –1.5 | –3.7 |
| BMNI_II0387 | BMEII0844 | outer membrane protein Omp31 | 177 | –1.5 | –3.0 |
| BMNI_II0117 | BMEII1114 | Flagellar biosynthesis protein FlhB | 235 | –1.5 | –2.3 |
| BMNI_I1173 | BMEI0785 | lytic transglycosylase | 111 | –1.4 | –1.7 |
| BMNI_I0560 | BMEI1384 | Helix-turn-helix domain-containing protein | 133 | –1.1 | –1.6 |
| BMNI_I0561 | BMEI1383 | Transcriptional regulator | 160 | –1.1 | –1.6 |
| BMNI_II0284 | BMEII0953 | respiratory nitrate reductase subunit gamma | 157 | 0.6 | 2.45 |
| BMNI_I1651 | BMEI0320 | GntR family transcriptional regulator | 95 | 0.6 | 2.2 |
| BMNI_II0145 | BMEII1088 | flagellar basal body rod protein FlgC | 88 | 0.8 | 3.47 |
| BMNI_II0377 | BMEII0854 | Crp/FNR family transcriptional regulator | 152 | 0.6 | 2.1 |

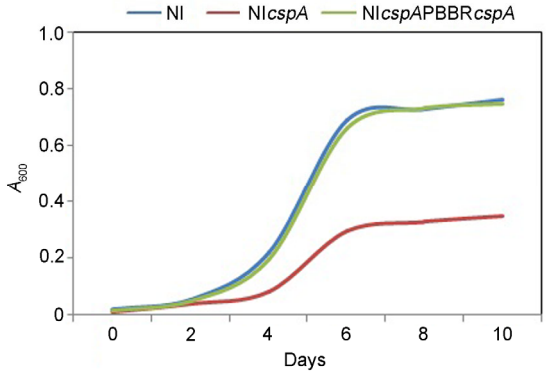


**Figure 2** Growth curves of the parental *B. melitensis* NI, mutant NIΔ*cspA*, and complementation strain NIΔ*cspA* PBBR*cspA* in minimal media. The minimal medium contains only carbon and nitrogen as nutrient resources. Compared to the wild-type strain, the *cspA* mutant showed a decreased $A_{600}$ value. The gene complementation of the *cspA* mutant resumed the $A_{600}$ level of the parental *B. melitensis* NI. A curve of the optical density values at $A_{600}$ determined at different time points reflects the growth dynamics of a bacterial strain in the culture medium over the different time points.

### *cspA* influences the expression of *virB* genes and then influences the function of T4SS

The *virB* operon encodes T4SS, one of the most important virulence structures essential for *B. melitensis* intracellular survival and infection. This gene was found to be down-regulated in NIΔ*cspA*, which may contribute to the attenuation of NIΔ*cspA* in J774.A1 macrophages and in mice. In order to further detect the function of T4SS in NIΔ*cspA*, NIΔ*cspA* and *B. melitensis* NI expressing the TEM1-VceC fusion protein were then used to infect J774.A1 macrophages for detection of VceC protein translocation (an effector protein of the *Brucella* T4SS translocated into macrophages) (de Jong et al., 2008). Translocation of the TEM1-VceC fusion protein by *B. melitensis* into host cells loaded with the fluorescent beta-lactamase substrate (CCF4/AM) was expected to lead to a shift in color of the cells from green to blue. As shown in Figure 3, at 6 h post-infection, we observed translocation of TEM1-VceC into macrophages infected by *B. melitensis* NI; the percentage of blue cells was approximately 1%, and the positive judgment standard was >0.5% of the number of blue cells divided by the number of total cells (de Jong et al., 2008) (Figure 3A). However, we did not detect blue cells loaded with the TEM1-VceC fusion protein among NIΔ*cspA* (Figure 3B) and a *B. melitensis* 16M *virB* mutant infected cells (Figure 3C). Western blotting of *B. melitensis* NI, NIΔ*cspA*, and the *virB* mutant expressing TEM1-VceC showed that all strains expressed the fusion protein at equivalent levels (Figure 3D). These results indicate that CspA influences the function of *B. melitensis* T4SS.

## DISCUSSION

CspA was previously shown to be linked to the regulation of both normal growth and stress adaptation processes as well as to the survival of stationary-phase cells in some organisms (Graumann et al., 1997; Phadtare and Inouye, 2004; Schmid et al., 2009). Our previous studies showed that the *cspA* gene deletion mutant NIΔ*cspA* was attenuated *in vivo* and *in vitro* (Wang et al., 2014), and the data from the present study clearly demonstrate that NIΔ*cspA* exhibits transcriptional differences compared with *B. melitensis* NI.

Survival and replication inside host cells by *B. melitensis* requires T4SS. Mutants of *Brucella* lacking functional T4SS are highly attenuated in macrophages and mouse models (O'Callaghan et al., 1999; den Hartigh et al., 2004). One remarkable result of our study is that the transcriptions of T4SS encoding genes were significantly down-regulated in NIΔ*cspA*. T4SS is required for *Brucella* to evade fusion of endosome vesicles with lysosomes (Sieira et al., 2000; Celli

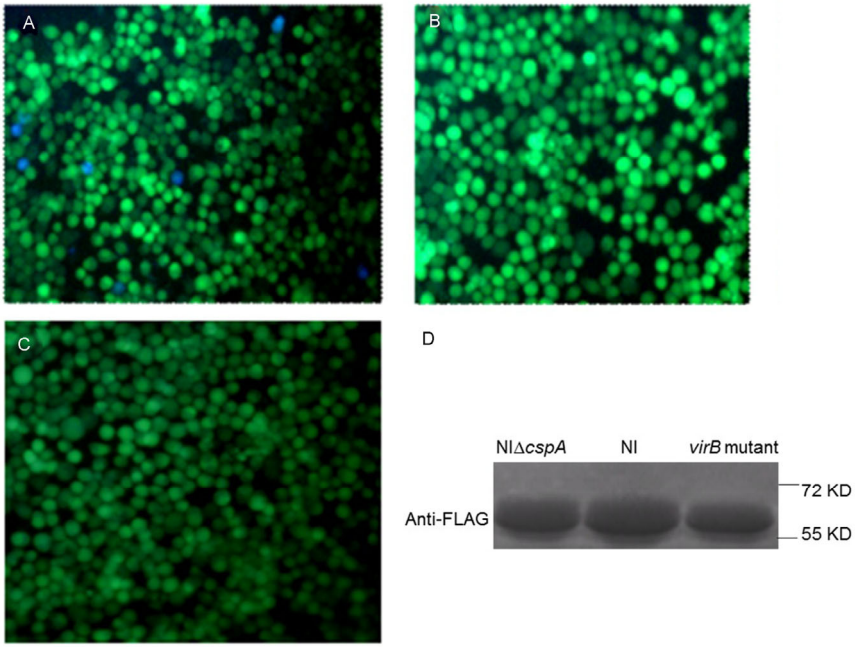


**Figure 3** Translocation of TEM1-VceC into J774.A1 macrophages. *B. melitensis* NI (A), NIΔ*cspA* (B), and *virB* mutant (C) expressing FLAG–TEM1 fused to VceC protein. Cells in which translocation of the fusion protein has occurred appear as blue. Results are from a representative individual experiment repeated three times independently. D, Western blot showing equal expression levels of FLAG-TEM1 fusion proteins in parent strain *B. melitensis* NI, NIΔ*cspA*, and *virB* mutant. Proteins were detected using anti-FLAG antiserum.

et al., 2003; Celli et al., 2005) by secreting effector proteins, allowing the bacteria to reside and replicate in vacuoles (Sieira et al., 2000). VceC was reported to be translocated into macrophages by *Brucella* T4SS (de Jong et al., 2008). The secretion of VceC by T4SS in NIΔ*cspA* was tested using the TME1 reporting system. The results suggested that the function of T4SS in NIΔ*cspA* is influenced by deletion of the *cspA* gene.

Expression of the *B. melitensis virB* locus is influenced by several transcriptional regulators, including LuxR-type regulatory protein VjbR (Delrue et al., 2005) and BabR (also called BlxR) (Rambow-Larsen et al., 2008), MucR (Dong et al., 2013; Mirabella et al., 2013), and the histidine utilization regulator HutC (Sieira et al., 2010). In our study, the *virB* operon, several OMPs, and flagellar genes were found to be differentially expressed in NIΔ*cspA*. Except for VjbR, transcriptional regulators belonging to the GntR families were also down-regulated in NIΔ*cspA*, which have been implicated in the regulation of virulence genes in several other pathogenic organisms (Haine et al., 2005). Similarly to the two-component regulator BvrR/BvrS, except genes involved in cell envelope modulation and carbon and nitrogen metabolism which were differentially expressed, several transcriptional regulators including VjbR, ExoR, and OmpR also showed lower expression in the *bvrR* mutant, suggesting the existence of a complex regulatory network involving the interplay of several transcriptional regulators in *Brucella* (Martinez-Nunez et al., 2010).

Moreover, our results showed that CspA played an important role in the metabolism of *Brucella*, resulting in the differential expression of metabolism-related genes and the reduced growth of NIΔ*cspA* in minimal media. For example, genes involved in pyruvate metabolism, which plays an important role in balancing energy metabolism (Martin et al., 2005), may also contribute to the virulence of *Brucella*.

Since CspA is important in *Brucella* virulence and metabolism regulation, further studies are required to analyze the CspA-mediated regulatory mechanisms. Since many genes have been found to be regulated by CspA, it may be possible to use bioinformatics and experimental methods to predict and identify the binding site(s) of CspA. Additionally, how CspA interacts with and regulates this large number of *Brucella* genes must be examined. Instead of up- or down-regulating a large number of genes simultaneously, it is more likely that CspA regulates these genes through one or more defined pathways. The eventual discovery of the detailed CspA-regulated interaction networks will be critical for understanding *Brucella* pathogenesis and will support the rational design of preventive vaccines.

In conclusion, we evaluated the different phenomena associated with the virulence and metabolism of NIΔ*cspA* and its parental strain NI. This study provides important information for understanding the detailed CspA-regulated interaction networks and *Brucella* pathogenesis.

## MATERIALS AND METHODS

### Bacterial strains and media

*Brucella melitensis* NI is an epidemic strain isolated from

an aborted bovine fetus in China. The complete NI genomes were sequenced and have GenBank accession numbers of CP002931 and CP002932 (Liu et al., 2012). NIΔ*cspA* is a *cspA* gene deletion strain constructed previously through a novel positive/negative selection method in our lab. NIΔ*cspA* PBBR*cspA* is the complementation strain of NIΔ*cspA* (Wang et al., 2014). The *B. melitensis* 16M *virB* mutant was previously constructed in our lab. All *Brucella* strains were routinely grown in tryptic soy broth (TSB) or tryptic soy agar at 37°C. All work with live virulent *B. melitensis* strains was performed in biosafety level three facilities at China Agricultural University.

### Bacterial growth and RNA preparation

*Brucella melitensis* NI and NIΔ*cspA* were grown with 100 mL of TSB, pH 7.3 (BD Biosciences, USA) in a water-bath shaker (180 r $min^{-1}$) at 37°C until the cells reached the exponential phase (approximately $10^9$ colony-forming units (CFU) $mL^{-1}$). The bacteria cultures were collected and centrifuged. After centrifugation, the supernatants were removed and the RNA protect Bacteria Reagent (Qiagen, Germany) was added to the pellets to prevent RNA degradation. The RNA samples from *B. melitensis* NI and NIΔ*cspA* for Solexa/Illumina sequence were isolated and purified using the RNeasy Mini System (Qiagen). RNAs was eluted from the column using RNase-free water. Total RNA was incubated with DNase (Ambion, USA) and then purified using two phenol-chloroform extractions and one chloroform extraction. RNA was resuspended in RNAase-free TE buffer (10 mmol $L^{-1}$ Tris, 1 mmol $L^{-1}$ ethylene diamine tetraacetic acid (EDTA); pH 8.0; Ambion). The purity and integrity of RNAs were assessed using the 2100 Bioanalzyer (Agilent Technologies, USA). *B. melitensis* mRNA was enriched by removing 16S and 23S rRNAs from two 5-μg aliquots of total RNA using the MicrobExpress Bacterial mRNA purification Kit (Ambion). As⩽5 μg total RNA was treated per reaction, a separate enrichment reaction was performed for each RNA sample to enrich the RNA volume for subsequent experiments. The mRNA sample was assessed using the 2100 Bioanalyzer to confirm the reduction of 16S and 23S rRNAs prior to the preparation of cDNA fragment libraries.

### cDNA library preparation and sequencing using the Illumina Genome Analyzer

RNA-Seq is a recently developed approach for transcriptome profiling that uses deep-sequencing technologies. In general, each mRNA sample was interrupted into short fragments. Short fragments were purified using the QiaQuick PCR Purification kit (Qiagen) and resolved with EB buffer for end-preparation and the addition of poly (A). Next, the DNA libraries were prepared according to the Illumina protocols. Sequencing was carried out on an Illumina HiSeqTM 2000 platform (USA). The 90-base pair raw sequence data were generated using the Illumina Genome Analyzer II system. The raw sequence data was filtered by removing reads containing adaptor sequences, consisting of >5% ambiguous residues (Ns), or containing a base quality of <5.

### Identification of transcribed annotated coding sequences and intergenic region

The *B. melitensis* NI genome sequences were downloaded from the National Center for Biotechnology Information (ftp://ftp.ncbi.nlm.nih.gov/genomes/). We then aligned the reads to the *B. melitensis* NI genome and annotated the gene sets using the Short Oligonucleotide Analysis Package (SOAP) (Li et al., 2009). cDNAs with matches to the reference genome of >80% were retained for further analysis. Gene expression was quantified as reads per kilobase of coding sequence per million reads (RPKM) algorithm. Genes were considered to be differentially expressed if the difference in RPKM values between the two samples was ⩾2.0-fold (i.e., $\log_2$ ratio >1.0) and the *P* value was <0.05 (Mortazavi et al., 2008).

### Cluster of orthologous group (COG) category and pathway analysis of *cspA*-dependent genes

The next-generation sequencing method revealed the transcription levels of genes in NIΔ*cspA* and NI. All possible *cspA*-dependent genes were identified using statistical methods. COG annotations for the chosen genes were obtained from NCBI COG database (http://www.ncbi.nlm.nih.gov/COG/). The program OntoCOG was used for the COG enrichment test as previously described (Lin et al., 2011).

### *In vitro* growth characteristics in minimal medium

To monitor extracellular growth, we inoculated one colony from each strain (NIΔ*cspA*, NIΔ*cspA* PBBR*cspA*, and *B. melitensis* NI) into 5 mL of TSB medium and grown to mid-log phase in a shaking incubator at 37°C. These cultures were then adjusted to the same concentration (CFU $mL^{-1}$) and subsequently used for growth curve analysis. Briefly, a 20 μL sample of each strain was inoculated into 5 mL of minimal medium (0.5% lactic acid, 3% glycerol, 0.75% NaCl, 1% $K_2HPO_4$, 0.01% $Na_2S_2O_3 \cdot 5H_2O$, 10 μg $mL^{-1}$ $MgCl_2 \cdot 6H_2O$, 0.1 μg $mL^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.1 μg $mL^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.21 μg $mL^{-1}$ thiamine·HCl, 0.2 μg $mL^{-1}$ nicotinic acid, 0.04 μg $mL^{-1}$ calcium pantothenate, 0.001 μg $mL^{-1}$ biotin, 5 mg $mL^{-1}$ glutamate; pH 6.8–7.0 with NaOH), followed by incubation in a shaking incubator at 37°C. The $A_{600}$ was determined at 0 h and 2, 4, 6, 8, and 10 d after inoculation. Growth characteristics were evaluated by analyzing the growth curves of NIΔ*cspA* at different time points.

### Effector translocation assay

To determine the secretion function of T4SS in *B. melitensis* NIΔ*cspA*, an effector translocation assay was performed as described previously (de Jong et al., 2008). Briefly, the

TEM1-VceC fusion plasmid was introduced into *B. melitensis* NI, a *B. melitensis* 16M *virB* mutant, and NIΔ*cspA* by electroporation. The TEM1-VceC fusion plasmid was constructed by our lab, and the original pZL1790-TME1 plasmid was donated by Prof. Zhaoqing Luo. Expression of the fusion protein TEM1-VceC in *B. melitensis* was confirmed by western blotting using anti-Flag antibodies. For the translocation assay, $6\times10^4$ J774.A1 mouse macrophages were seeded into 96-well plates and infected with *B. melitensis* NI, *B. melitensis* 16M *virB* mutant, and NIΔ*cspA* expressing TEM1-VceC fusion protein at a multiplicity of infection of 500∶1. Cells were incubated for 20 min at 37°C in 5% $CO_2$ and washed twice with phosphate-buffered saline and 0.1 mL of fresh DMEM plus 1 mmol $L^{-1}$ IPTG in each well of the plate, and the plates were incubated at 37°C in 5% $CO_2$. At 6 h post-infection, the cells were washed once with Hank's balanced salt solution (Gibco, Grand Island, NY) and loaded with a solution containing the fluorescent substrate CCF4/AM at a final concentration of 1 mmol $L^{-1}$ for 1.5 h at room temperature using the standard loading protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). Next, the translocation of TEM1-VceC fusion protein into macrophages was analyzed by fluorescence microscopy.

### Quantitative real-time PCR

To confirm the RNA-Seq results, 18 up- or down-regulated genes from the RNA-Seq analysis were selected and RT-PCR and RT-qPCR were carried out to confirm the gene expression changes of these 18 genes. PCR primers were designed using Primer 5.0 software (Primer-E Ltd., Plymouth, UK) and are listed in Table S2. The same experimental protocols were used to culture both wild-type NI and NIΔ*cspA* and extract RNA samples as RNA-seq. Immunofluorescence analysis was performed with SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using the 7500 Real Time PCR System (Applied Biosystems) as previously described (Liu et al., 2012). The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with the 16S rRNA gene of *B. melitensis* as a reference.

### Statistical analysis

Differences between the means of gene expression for the experimental and control groups were analyzed by the Student's unpaired *t*-test (equal sample sizes, equal variance) using SPSS 18.0 (SPSS, Inc., Chicago, IL). For the RNA-seq study, the *P*-values with false discovery rate multi-test adjustment were used to identify differentially expressed genes in the experimental groups compared to in the control groups. The false discovery rate *P*-value ⩽0.001 and the absolute value of $\log_2$ ratio ⩾1 (i.e., 2-fold change) were used as thresholds to identify genes showing statistically significant gene expression changes. For the RT-PCR study, a *P*-value <0.05 was considered to be statistically significant. The Student's unpaired *t*-test was also used to analyze the bacterial survival rates under minimal media conditions.

**Compliance and ethics** *The author(s) declare that they have no conflict of interest.*

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Bae, W., Jones, P.G., and Inouye, M. (1997). CspA, the major cold shock protein of *Escherichia coli*, negatively regulates its own gene expression. J Bacteriol 179, 7081–7088.

Briones, G., Inon, D.I.N., Roset, M., Vigliocco, A., Paulo, P.S., and Ugalde, R.A. (2001). *Brucella abortus* cyclic beta-1,2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. Infect Immun 69, 4528–4535.

Caswell, C.C., Gaines, J.M., Ciborowski, P., Smith, D., Borchers, C.H., and Roux, C.M. (2012). Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. Mol Microbiol 85, 345–360.

Celli, J., and Gorvel, J.P. (2004). Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr Opin Microbiol 7, 93–97.

Celli, J., Salcedo, S.P., and Gorvel, J.P. (2005). *Brucella* coopts the small GTPase Sar1 for intracellular replication. Proc Natl Acad Sci USA 102, 1673–1678.

Celli, J., de Chastellier, C., Franchini, D.M., Pizarro-Cerda, J., Moreno, E., and Gorvel, J.P. (2003). *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med 198, 545–556.

Croucher, N.J., and Thomson, N.R. (2010). Studying bacterial transcriptomes using RNA-seq. Curr Opin Microbiol 13, 619–624.

de Jong, M.F., Sun, Y.H., den Hartigh, A.B., van Dijl, J.M., and Tsolis, R.M. (2008). Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. Mol Microbiol 70, 1378–1396.

Delrue, R.M., Deschamps, C., Leonard, S., Nijskens, C., Danese, I., Schaus, J.M., Bonnot, S., Ferooz, J., Tibor, A., De Bolle, X., and Letesson, J.J. (2005). A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. Cell Microbiol 7, 1151–1161.

den Hartigh, A.B., Sun, Y.H., Sondervan, D., Heuvelmans, N., Reinders, M.O., Ficht, T.A., and Tsolis, R.M. (2004). Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect Immun 72, 5143–5149.

Dong, H., Liu, W., Peng, X., Jing, Z., and Wu, Q. (2013). The effects of MucR on expression of type IV secretion system, quorum sensing system and stress responses in *Brucella melitensis*. Vet Microbiol 166, 535–542.

Ermolenko, D.N., and Makhatadze, G.I. (2002). Bacterial cold-shock proteins. Cell Mol Life Sci 59, 1902–1913.

Fretin, D., Fauconnier, A., Kohler, S., Halling, S., Leonard, S., Nijskens, C., Ferooz, J., Lestrate, P., Delrue, R.M., Danese, I., Vandenhaute, J., Tibor, A., DeBolle, X., and Letesson, J.J. (2005). The sheathed flagellum of *Brucella melitensis* is involved in persistence in a murine model of infection. Cell Microbiol 7, 687–698.

Graumann, P., Wendrich, T.M., Weber, M.H., Schroder, K., and Marahiel, M.A. (1997). A family of cold shock proteins in Bacillus subtilis is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Mol Microbiol 25, 741–756.

Haine, V., Dozot, M., Dornand, J., Letesson, J.J., and De Bolle, X. (2006). NnrA is required for full virulence and regulates several *Brucella melitensis* denitrification genes. J Bacteriol 188, 1615–1619.

Haine, V., Sinon, A., Van Steen, F., Rousseau, S., Dozot, M., Lestrate, P., Lambert, C., Letesson, J.J., and De Bolle, X. (2005). Systematic targeted mutagenesis of *Brucella melitensis* 16M reveals a major role for

GntR regulators in the control of virulence. Infect Immun 73, 5578–5586.
He, Y. (2012). Analyses of *Brucella* pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. Front Cell Infect Microbiol 2, 2.
Jiang, W., Hou, Y., and Inouye, M. (1997). CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. J Biol Chem 272, 196–202.
Jiao, X., Sherman, B.T., Huang, D.W., Stephens, R., Baseler, M.W., Lane, H.C., and Lempicki, R.A. (2012). DAVID-WS: a stateful web service to facilitate gene/protein list analysis. Bioinformatics 28, 1805–1806.
Li, R., Yu, C., Li, Y., Lam, T.W., Yiu, S.M., Kristiansen, K., and Wang, J. (2009). SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25, 1966–1967.
Lin, Y., Xiang, Z.S., He, Y. (2011). Towards a semantic web application: ontology-driven ortholog clustering analysis. International Conference on Biomedical Ontologies (ICBO), Buffalo, USA, 33–40.
Liu, W., Jing, Z., Ou, Q., Cui, B., He, Y., and Wu, Q. (2012). Complete genome sequence of *Brucella melitensis* biovar 3 strain NI, isolated from an aborted bovine fetus. J Bacteriol 194, 6321.
Liu, W., Dong, H., Liu, W., Gao, X., Zhang, C., and Wu, Q. (2012). OtpR regulated the growth, cell morphology of *B. melitensis* and tolerance to beta-lactam agents. Vet Microbiol 159, 90–98.
Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method Methods 25, 402–408.
Loisel-Meyer, S., Jimenez, D.B.M., Basseres, E., Dornand, J., Kohler, S., Liautard, J.P., and Jubier-Maurin, V. (2006). Requirement of norD for *Brucella suis* virulence in a murine model of *in vitro* and *in vivo* infection. Infect Immun 74, 1973–1976.
Lowry, J.E., Isaak, D.D., Leonhardt, J.A., Vernati, G., Pate, J.C., and Andrews, G.P. (2011). Vaccination with *Brucella abortus* recombinant *in vivo*-induced antigens reduces bacterial load and promotes clearance in a mouse model for infection. PLoS One 6, e17425.
Martin, M.G., Magni, C., de Mendoza, D., and Lopez, P. (2005). CitI, a transcription factor involved in regulation of citrate metabolism in lactic acid bacteria. J Bacteriol 187, 5146–5155.
Martinez-Nunez, C., Altamirano-Silva, P., Alvarado-Guillen, F., Moreno, E., Guzman-Verri, C., and Chaves-Olarte, E. (2010). The two-component system BvrR/BvrS regulates the expression of the type IV secretion system VirB in *Brucella abortus.* J Bacteriol 192, 5603–5608.
Martirosyan, A., Moreno, E., and Gorvel, J.P. (2011). An evolutionary strategy for a stealthy intracellular *Brucella* pathogen. Immunol Rev 240, 211–234.
Mirabella, A., Terwagne, M., Zygmunt, M.S., Cloeckaert, A., De Bolle, X., and Letesson, J.J. (2013). *Brucella melitensis* MucR, an orthologue of Sinorhizobium meliloti MucR, is involved in resistance to oxidative, detergent, and saline stresses and cell envelope modifications. J Bacteriol 195, 453–465.
Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5, 621–628.
O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., and Ramuz, M. (1999). A homologue of the Agrobacterium tumefaciens VirB and Bordetella pertussis Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol Microbiol 33, 1210–1220.
Phadtare, S. (2004). Recent developments in bacterial cold-shock response. Curr Issues Mol Biol 6, 125–136.
Phadtare, S., and Inouye, M. (2004). Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of *Escherichia coli*. J Bacteriol 186, 7007–7014.
Pinto, A.C., Melo-Barbosa, H.P., Miyoshi, A., Silva, A., and Azevedo, V. (2011). Application of RNA-seq to reveal the transcript profile in bacteria. Genet Mol Res 10, 1707–1718.
Rambow-Larsen, A.A., Rajashekara, G., Petersen, E., and Splitter, G. (2008). Putative quorum-sensing regulator BlxR of *Brucella melitensis* regulates virulence factors including the type IV secretion system and flagella. J Bacteriol 190, 3274–3282.
Rodriguez, M.C., Viadas, C., Seoane, A., Sangari, F.J., Lopez-Goni, I., and Garcia-Lobo, J.M. (2012). Evaluation of the effects of erythritol on gene expression in *Brucella abortus*. PLoS One 7, e50876.
Schmid, B., Klumpp, J., Raimann, E., Loessner, M.J., Stephan, R., and Tasara, T. (2009). Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl Environ Microbiol 75, 1621–1627.
Seleem, M.N., Boyle, S.M., and Sriranganathan, N. (2008). *Brucella*: a pathogen without classic virulence genes. Vet Microbiol 129, 1–14.
Sieira, R., Comerci, D.J., Sanchez, D.O., and Ugalde, R.A. (2000). A homologue of an operon required for DNA transfer in Agrobacterium is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 182, 4849–4855.
Sieira, R., Arocena, G.M., Bukata, L., Comerci, D.J., and Ugalde, R.A. (2010). Metabolic control of virulence genes in *Brucella abortus*: HutC coordinates virB expression and the histidine utilization pathway by direct binding to both promoters. J Bacteriol 192, 217–224.
Sola-Landa, A., Pizarro-Cerda, J., Grillo, M.J., Moreno, E., Moriyon, I., Blasco, J.M., Gorvel, J.P., and López-Goñi. I. (1998). A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol 29, 125–138.
Tian, M., Qu, J., Han, X., Zhang, M., Ding, C., Ding, J., Chen, G., and Yu, S. (2013). Microarray-based identification of differentially expressed genes in intracellular *Brucella abortus* within RAW264.7 cells. PLoS One 8 e67014.
Wang, F., Hu, S., Liu, W., Qiao, Z., Gao, Y., and Bu, Z. (2011). Deep-sequencing analysis of the mouse transcriptome response to infection with *Brucella melitensis* strains of differing virulence. PLoS One 6, e28485.
Wang, Z., Wang, S., and Wu, Q. (2014). Cold shock protein A plays an important role in the stress adaptation and virulence of *Brucella melitensis*. FEMS Microbiol Lett 354, 27–36.
Wu, Q., Pei, J., Turse, C., and Ficht, T.A. (2006). Mariner mutagenesis of *Brucella melitensis* reveals genes with previously uncharacterized roles in virulence and survival. BMC Microbiol 6, 102.



## SUPPORTING INFORMATION

**Table S1** *cspA*-dependent differentially expressed genes in NIΔ*cspA*

**Table S2** RT-PCR primers used in this study

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